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TITLE: Development of Specific Inhibitors for Breast Cancer-Associated Variants of ErbB2

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14. ABSTRACT
The receptor tyrosine kinase HER2/ErbB2 is one of the most highly mutated tyrosine kinases in breast cancer. Several mutations within the ErbB2 kinase domain have been identified in breast cancer patients, but in most cases it is not known whether these mutations increase kinase activity and signaling. The first goal of this project is to provide data linking the mutations to their degree of kinase activation. In the first year of this project, we have expressed and purified the wild-type ErbB2 kinase domain and four cancer-associated mutants, one of which (G776C) has not previously been reported. We have developed a kinase assay to be used in inhibitor screening. All four mutants are hyperactivated (relative to wild-type ErbB2), as assessed by in vitro kinase assays and cellular assays. In the next phase of the project, we will screen inhibitors identified by our collaborator, Dr. Robert Rizzo.

15. SUBJECT TERMS
Tyrosine kinase, ErbB2, inhibitors, virtual ligand screening, kinase assay

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1. INTRODUCTION

One of the most highly mutated tyrosine kinases in breast cancer is the tyrosine kinase ErbB2. The central hypothesis of this project is that some of the ErbB2 mutations uncovered by breast cancer genome sequencing lead to unregulated kinase activity. The research team consists of two principal investigators at Stony Brook University. Dr. Miller has expertise in tyrosine kinase signaling, and Dr. Rizzo is an expert in computational modeling procedures. The *first objective* is to test the cellular and *in vitro* kinase activity of all of the ErbB2 mutants. The *second objective* is to use computational screening methods to identify compounds that can selectively target and inhibit those ErbB2 mutants verified as active. The *third objective* is to experimentally test a subset of the most promising compounds for the ability to experimentally inhibit specific ErbB2 mutants. This report will focus on the progress done in Dr. Miller's laboratory.

2. KEYWORDS

Tyrosine kinase, ErbB2/HER2, enzyme inhibition, protein phosphorylation, computational modeling, virtual screening.

3. ACCOMPLISHMENTS

What were the major goals of the project?

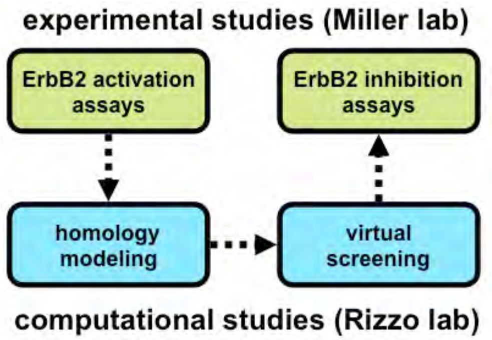
From approved Statement of Work:

Specific Aim 1: Testing cellular activity of ErbB2 mutants.
Major Task 1: Express ErbB2 mutants in NIH3T3 cells (Months 1-4)
Major Task 2: Measurements of ErbB2 activity (Months 5-8)
Milestone #1: Identification of activated ErbB2 mutants. This will enable work on Specific Aims 2 and 3 to proceed concurrently. (Month 8)
Specific Aim 2: Biochemical studies of activated ErbB2 mutants
Major Task 3: Express and purify ErbB2 mutants (Months 8-12)
Major Task 4: In vitro activity measurements (Months 9-15)
Specific Aim 3: Identifying inhibitors of ErbB2 mutants.*
Major Task 5: Produce ErbB2 structures for drug-lead identification (Months 1-12)*
*Milestone #2: Production of computationally-derived pdb files of the structures of activated forms of ErbB2. (Month 12)**
Major Task 6: Virtual screening and experimental validation (Months 8-24)*
*Milestone #3: Identification of inhibitors for each activated form of ErbB2. (Month 24)**
*Milestone #4: Manuscript on combined use of experimental and computational approaches to identify ErbB2 inhibitors . (Month 24)**

*These aims/tasks describe work to be done in Dr. Rizzo's laboratory, and will not be discussed in detail in this report.

What was accomplished under these goals?

The schematic diagram below outlines the overall research strategy of this collaborative project.



We have made significant progress in our characterization of ErbB2 mutants. We are on target with respect to the timeline described in our Statement of Work. In summary, we have identified the activated cancer-associated ErbB2 mutants that will be used for drug screening, and we have established enzyme assays that will be suitable for screening.

<u>Specific Aim 1: Testing cellular activity of ErbB2 mutants</u>.

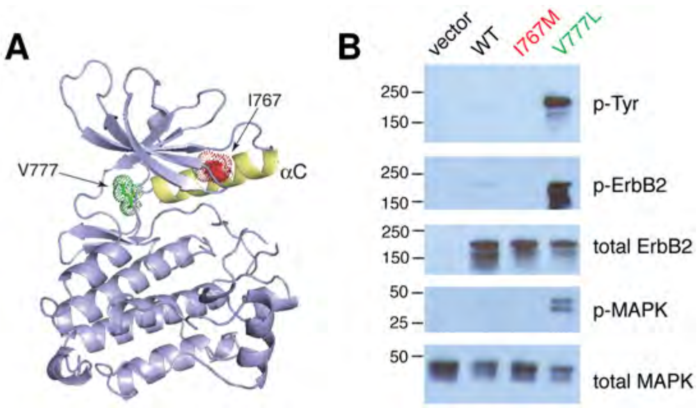
Using site-directed mutagenesis, we have produced the following cancer-associated mutant forms of ErbB2:

D769Y
V777L
P780 insertion: GSP
L755M
L755W
G776V
G776L
G776C
G776S
I767M

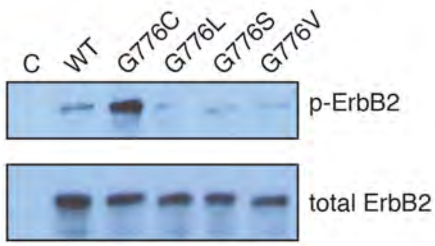
A change from the original proposal is that we have elected not to pursue several of the ErbB2 mutants listed in the proposal. This is because several of the mutants were characterized in a recent publication (Bose *et al.*, *Cancer Discovery*, 3: 224-237, 2013). This publication was helpful to us and accelerated our work, since Bose *et al.* validated our work on the V777L mutant, and identified two other mutants (D769Y and the P780 insertion) that are hyperactivated relative to wild-type ErbB2. As described below, we will pursue virtual ligand screening of these two mutants. The other mutations listed above have not been characterized prior to our work.

As proposed, we have expressed the ErbB2 mutants in mammalian cells and tested their activities by Western blotting experiments. We expressed wild-type and mutant forms of ErbB2 in NIH3T3 cells. After verifying equivalent expression, we carried out Western blotting to measure kinase activity. We used activation-state specific antibodies for ErbB2 and for the key downstream substrate MAPK. The following mutants have tyrosine kinase activity that is consistently higher than WT ErbB2: V777L, D769Y, P780 insertion, and G776C. Representative data for I767M, V777L and the multiple mutations at G776 are shown below. Downstream

signaling is also enhanced in cells expressing these mutants, as shown by increased phosphorylation of MAP kinase.



Cancer-associated mutations in ErbB2. (A) Crystal structure of the ErbB2 kinase catalytic domain (PDB code 3PP0) in the active form. Helix αC is shown in yellow. The sidechains of I767 (red) and V777 (green) are shown in dotted surface representations. (B) Wild-type or mutant forms of ErbB2 were expressed in NIH3T3 cells. Cell lysates were examined by Western blotting with the indicated antibodies. Similar results were obtained in three separate experiments.

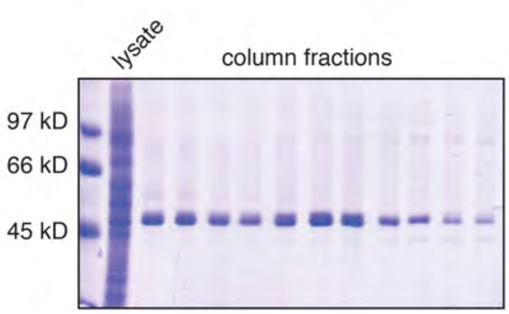


ErbB2 mutations at codon 776. Wild-type or mutant forms of ErbB2 were expressed in NIH3T3 cells. Cell lysates were examined by Western blotting with the indicated antibodies. Similar results were obtained in three separate experiments.

Thus, we have achieved Milestone #1 (Identification of activated ErbB2 mutants), and work on Specific Aims 2 and 3 has been proceeding on schedule.

Specific Aim 2: Biochemical studies of activated ErbB2 mutants.

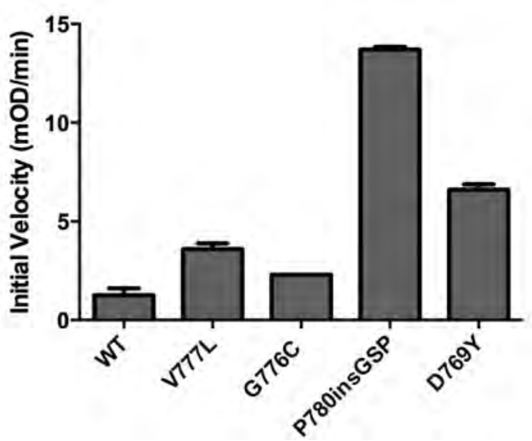
We generated recombinant baculoviruses encoding wild-type ErbB2 and each mutant form of ErbB2 (V777L, D769Y, P780 insertion, and G776C) using the Bac-to-Bac system. We scaled up the baculoviruses and used them to infect Sf9 cells in one-liter spinner flasks. We purified the proteins using metal affinity, ion exchange, and gel filtration chromatography. A representative purification of WT ErbB2 is shown below. This was a very gratifying result, as unanticipated obstacles often arise during protein expression and purification.



Purification of wild-type ErbB2. Sf9 cells (600 ml) were infected with recombinant ErbB2 baculovirus, and cells were harvested and lysed after 3 days of infection. ErbB2 was purified using Ni-NTA chromatography and analyzed by SDS-PAGE. Detection was with Coomassie blue staining.

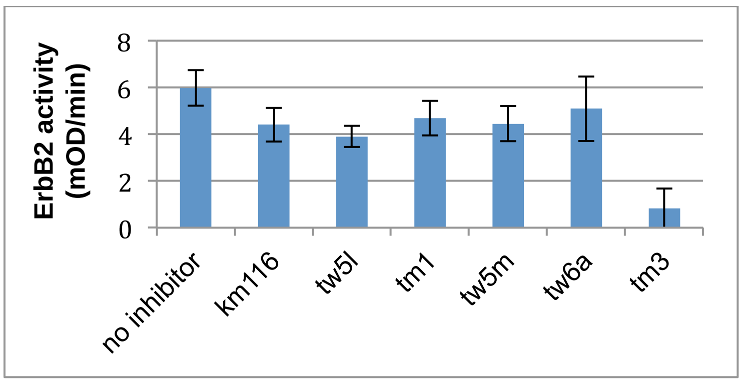
We measured enzyme activity using two assays: (1) a continuous spectrophotometric assay. In this assay, the production of ADP is coupled to the oxidation of NADH, which is measured as a reduction in absorbance at 340 nm. Enzyme progress curves were monitored by absorbance at 340 nm in a Molecular Devices UV/Vis microplate reader, and experiments were carried out with varying concentrations of ATP and peptide substrates. (2) A radioactive kinase assay, using [^{32}P]-ATP and peptide substrates. Our initial data (shown below) confirm the results of the cellular assays; all four variants of ErbB2 show enhanced kinase activity relative to the wild-

type protein. We are in the process of determining the kinetic parameters V_{max} and K_m for both ATP and peptide by fitting initial rate data to the Michaelis-Menten equation.



Activities of purified ErbB2 variants. The activities of purified forms of ErbB2 (wild-type or cancer-associated mutants) toward a synthetic peptide substrate were analyzed using the coupled spectrophotometric assay.

We have also adapted the spectrophotometric assay so that it can be used for screening potential inhibitors derived from Dr. Rizzo's computational virtual ligand screening. We have established conditions with fixed concentrations of peptide and ATP substrates so that the screening can be done in a 96-well format. Thus, we are in a good position to move ahead with Specific Aim 3, in collaboration with Dr. Rizzo.



ErbB2 inhibitor screen. The activity of purified ErbB2 was analyzed using the coupled spectrophotometric assay. In this preliminary experiment, assay conditions were tested with 6 newly-developed RTK inhibitors (kind gift of Dr. Markus Seeliger, Stony Brook University). The compounds were tested at 10 μM concentrations. Compound tm3 showed significant inhibition of WT ErbB2.

What opportunities for training and professional development has the project provided?

This was not explicitly a component of the project. However, progress on Specific Aims 1 and 2 has enabled the training of Mr. Steven Collins, a graduate student in Biochemistry and Structural Biology at Stony Brook, in the expression, purification, and characterization of ErbB2.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

(1) Complete kinetic analyses of wild-type and mutant forms of ErbB2. We will determine the kinetic parameters V_{max} and K_m for both ATP and peptide.

(2) Optimize and finalize conditions for inhibitor screening. We will test the following conditions: ATP and peptide concentrations; enzyme concentration; divalent cations; time of autophosphorylation.

(3) We will carry out initial rate ErbB2 measurements in the presence of inhibitors identified through virtual screening. This part of the project will proceed as the screening results become available from Dr. Rizzo's laboratory. Because Dr. Rizzo and colleagues will carry out the computational modeling and virtual screening on each individual ErbB2 mutant, we anticipate that our screening will also be done one mutant at a time.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

This project will add to our understanding of the molecular mechanisms involved in breast cancer. Genome sequencing efforts have outpaced our ability to predict the outcomes of mutations that are identified in breast cancer patients. Many breast cancer-associated mutations remain uncharacterized at the molecular level. For ErbB2, this project would bridge that gap by providing a dataset linking the mutations to their degree of kinase activation. This information will be important for understanding the molecular mechanisms involved in tumor initiation and progression.

The data provided by this project may also have a more direct impact on breast cancer treatment. First, activating ErbB2 mutations that represent *bona fide* drivers of breast cancer proliferation could potentially be used as diagnostic markers. Second, the dataset will be relevant to anticancer therapeutics (agents in current use, as well as novel agents to be developed in the future). Mutated cancer genes represent potential "Achilles' heels" that can be exploited for drug discovery. The identification of driver mutations opens the possibility of tailoring the treatment of a cancer patient based on a specific tumorigenic profile. For example, in non-small-cell lung cancer, specific EGFR mutations render tumors vulnerable to the kinase inhibitors gefitinib (Iressa) and erlotinib (Tarceva). Our well-validated computational approach will identify candidate inhibitors for the activated ErbB2 mutants.

We have obtained preliminary data showing that ErbB2 kinase is activated by the cancer-associated V777L mutation. If corroborated by further studies, this could have an impact on current treatment strategies. The two marketed ErbB2 inhibitors, trastuzumab (Herceptin) and lapatinib (Tykerb), are approved for patients whose tumors *overexpress* ErbB2. Our data introduce the possibility that a *mutation* in ErbB2 (V777L) could lead to deregulated signaling, even in tumors that do not overexpress ErbB2. Thus, it is conceivable that trastuzumab or lapatinib would also be beneficial to patients with this mutation.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS: There were no significant changes in the project or its direction, in expenditures, or in biohazards. As noted above under "Accomplishments," there was a minor change in the identity of some ErbB2 mutants to be tested.

6. PRODUCTS: Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING INSTITUTIONS:

What individuals have worked on the project?

Name:	W. Todd Miller
Project Role:	PI
Researcher identifier:	NIH Commons: millertodd
Nearest person month worked:	1
Contribution to Project:	Dr. Miller designed experiments, analyzed data, and coordinated efforts with Dr. Rizzo and colleagues.

Name:	Stephen J. Collins
Project Role:	Graduate student
Researcher identifier:	Stony Brook ID#109272847
Nearest person month worked:	12
Contribution to Project:	Mr. Collins designed and carried out ErbB2 experiments and analyzed data.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Award:
This grant agreement is a joint proposal with the following log number and the respective award number:
Log# BC132617P1
Grant Agreement Number: W81XWH-14-1-0420
Recipient: The Research Foundation of State University of New York, State University Stony Brook
Principal Investigator: Robert Rizzo

As outlined in the Contract, Dr. Rizzo will submit an independent annual report.

9. APPENDICES: Nothing to Report.